DETECTION OF A LYSOSOMAL CARBOXYPEPTIDASE AND A LYSOSOMAL DIPEPTIDASE IN HIGHLY-PURIFIED DIPEPTIDYL AMINOPEPTIDASE I (CATHEPSIN C) AND THE ELIMINATION OF THEIR ACTIVITIES FROM PREPARATIONS USED TO SEQUENCE PEPTIDES

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Summary - The best preparations of dipeptidyl aminopeptidase I (DAP I) from beef spleen and rat liver were found to contain a carboxypeptidase ("catheptic carboxypeptidase C") and a dipeptidase ("Ser-Met dipeptidase"). Each had a pH optimum near 5.5, a resistance to sulfhydryl inhibitors, and a lysosomal origin. The carboxypeptidase, which was inhibited by diisopropylphosphorofluoridate (DFP), preferentially cleaved COOH-terminal residues adjacent to proline, as in angiotensin II and Z-Pro-Phe. No action was detected on Z-Pro-Phe-NH_2. The dipeptidase, which was separable by electrofocusing, was most active on Ser-Met, and showed no action on Z-Ser-Met, Ser-Met-NH_2, Ser-Met-Glu, Gly-Gly or Gly-Leu. Ser-Met dipeptidase was unaffected by DFP, but was strongly inhibited by EDTA. A metal requirement was not apparent, however. A simplified method is described for preparing DAP I as a sequencing reagent free of these contaminating activities.

Previous papers (1,2) from this laboratory have demonstrated that highly purified DAP I* from rat liver or bovine spleen degrades a wide variety of hormonal and non-hormonal polypeptides. Typically, DAP I catalyzes the removal of dipeptides, in sequence, from the NH_2 terminus of a polypeptide. Consequently, we, as well as others, are presently exploring the feasibility of using DAP I as a peptide sequencing reagent (3).

Earlier studies revealed that highly purified DAP I contains a small amount of dipeptidase activity (1). For example, Ser-Tyr and Ser-Met, following their release from β-corticotropin by DAP I, underwent a partial breakdown during extended digestion times. Similarly, Thr-Phe and Thr-Ser were attacked following their appearance in glucagon and secretin digests (2). This communication describes the properties of a carboxypeptidase, termed "catheptic carboxypeptidase C," and a dipeptidase, termed "Ser-Met dipeptidase." Both were found in highly purified DAP I prepared from either beef spleen or rat liver. Methods are described for the removal or selective inactivation of these contaminating enzymes.

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*Abbreviations: DAP I, dipeptidyl aminopeptidase I; DFP, diisopropylphosphorofluoridate; PCMS, p-chloromercuriphenyl sulfonate; Z-, N^{α}-benzyloxycarbonyl-; CPC, carboxypeptidase C.

METHODS AND MATERIALS

Preparation and Assay of DAP I - The method of purification was basically that of Metrione, Neves, and Fruton (4) and involves an acid extraction of beef spleen, fractionation with ammonium sulfate, heat treatment, and column chromatography on Sephadex G-200, DEAE-cellulose, and CM-cellulose. The method has also been applied successfully to the purification of DAP I from rat liver (1). In that study, as well as in this report, DAP I was assayed by a direct, recording, fluorometric technique (5). One unit of enzyme is the amount hydrolyzing 1 μmole of Gly-Phe-β-naphthylamide per min at pH 6.0 and 37°. 2-Mercaptoethylamine hydrochloride was incorporated at 10 mM to meet both the sulfhydryl (6) and halide (7) requirements of DAP I.

Assay of Carboxypeptidases - Catheptic carboxypeptidase C was assayed by combining 0.1 ml of enzyme with 0.1 ml of 0.4 M acetate buffer, pH 5.5, and 0.8 ml of 0.019 M Z-Pro-Phe adjusted to pH 5.5. The reaction mixture was incubated 60 min at 37° and was terminated by adding 1 ml of 10% trichloroacetic acid. The liberated phenylalanine was measured colorimetrically using the ninhydrin procedure of Moore and Stein (8). Cathepsin A, which likewise lacks a sulfhydryl requirement, was assayed in a similar system using Z-Glu-Tyr as the substrate (9,10). The SH-dependent activities of catheptic carboxypeptidases A and B were assayed on Z-Glu-Tyr and N^{α}-benzoyl-Gly-Arg, respectively (11-13). The enzyme (0.1 ml) was incubated with 0.9 ml of a solution containing 0.016 M substrate in 0.1 M citrate buffer-5 mM dithiothreitol. The former activity was assayed at pH 3.5 (which excludes cathepsin A) and the latter at pH 5.5.

Assay of Ser-Met Dipeptidase - Reaction mixtures contained equal volumes of enzyme and 6 mM Ser-Met in 0.2 M cacodylic acid-NaOH, pH 5.5. Aliquots (0.1 ml) were taken in time for color development with trinitrobenzenesulfonic acid (TNBS) according to Okuyama and Satake (14). As previously reported (15), Cu^{++} was incorporated to great advantage to prevent the peptide substrate from reacting with the reagent. Furthermore, the TNBS-copper reagent could be used to measure dipeptidase activity in the presence of Ampholine.

Analysis of Peptide Digests - The course of angiotensin II digestion by DAP I was monitored by thin layer chromatography on microcrystalline cellulose. Plates were developed with sec-butanol-3% NH_3 (75:30). The same system was used on a preparative scale for the isolation of split products for identification by amino acid analysis. Dipeptide digests were also analyzed by thin layer chromatography, except the plates were developed with n-butanol-formic acid-water (70:15:15). Products were visualized with a polychromic ninhydrin reagent (16).

Source of Substrates - α-L-$Asp^{1}(NH_2)$-angiotensin II (angiotensin II amide; hypertensin-CIBA) was a gift received from Dr. Albert J. Plummer of Ciba (Summit,

New Jersey). The preparation was indicated to contain a small percentage of the α-L-Asp^1 analogue formed as an equilibrium by-product of the synthesis of angiotensin II amide. All other peptide substrates were obtained from Fox Chemical Company or Cyclo Chemical Corporation, both of Los Angeles, California. The purity and identity of these preparations were confirmed by thin layer chromatography and amino acid analysis.

RESULTS

Degradation of Angiotensin II Amide - The time course analysis shown in Fig. 1a demonstrates that highly purified DAP I has potent angiotensinase activity on α-L-$Asp^1(NH_2)$-angiotensin II. Both the rat liver and beef spleen enzymes cleaved Asn-Arg and Val-Tyr, in sequence, from the NH_2 terminus of this hypertensive peptide, which is the identity of the spot appearing at zero time. The transient hexapeptide is visible between 0.5 and 2 min. The DAP I attack was complete within 5 min using approximately 2×10^{-4} μmole of DAP I (mol wt 200,000) per μmole of angiotensin II. Subsequently, however, the tetrapeptide product, Val-His-Pro-Phe, underwent an unexpected conversion to Val-His-Pro and free phenylalanine. Evidence was therefore sought for the presence of a contaminating peptidase.

Such evidence is shown in Fig. 1b where it was possible to selectively inhibit the cleavage of the COOH-terminal phenylalanine by pretreating the DAP I preparation with DFP. A sulfhydryl inhibitor, on the other hand, had the opposite effect. As indicated by the time course in Fig. 1c, DAP I was completely inhibited by 1 mM PCMS, whereas the attack at the COOH terminus by the contaminating peptidase was unaffected. Under these conditions, the degradation of angiotensin II was restricted to the removal of phenylalanine. In Fig. 1c, the heptapeptide product appears as a doublet, as does the substrate, $Asp^1(NH_2)$-angiotensin II, seen at zero time. The existence of the doublet is explained by the known presence of a small percentage of Asp^1-angiotensin II. For each doublet, the components were separated and shown to have the same amino acid composition after acid hydrolysis. As seen in Figs. 1a and 1b, the components of the angiotensin II doublet were degraded simultaneously.

Enzyme controls showed only a faint amount of ninhydrin reaction at the origin that was unchanged during the time course. Substrate controls showed angiotensin II to be stable in the absence of enzyme.

The COOH-terminal attack on $Asp^1(NH_2)$-angiotensin II cannot be attributed to the carboxypeptidase activity of cathepsin A (17). As illustrated in Fig. 2, cathepsin A was destroyed by the 40-min heat treatment used in the preparation of DAP I. Although a portion of the catheptic carboxypeptidase A and B activities survived the heat treatment, their sensitivity to sulfhydryl inhibitors distinguished them from the carboxypeptidase that contaminates DAP I preparations.

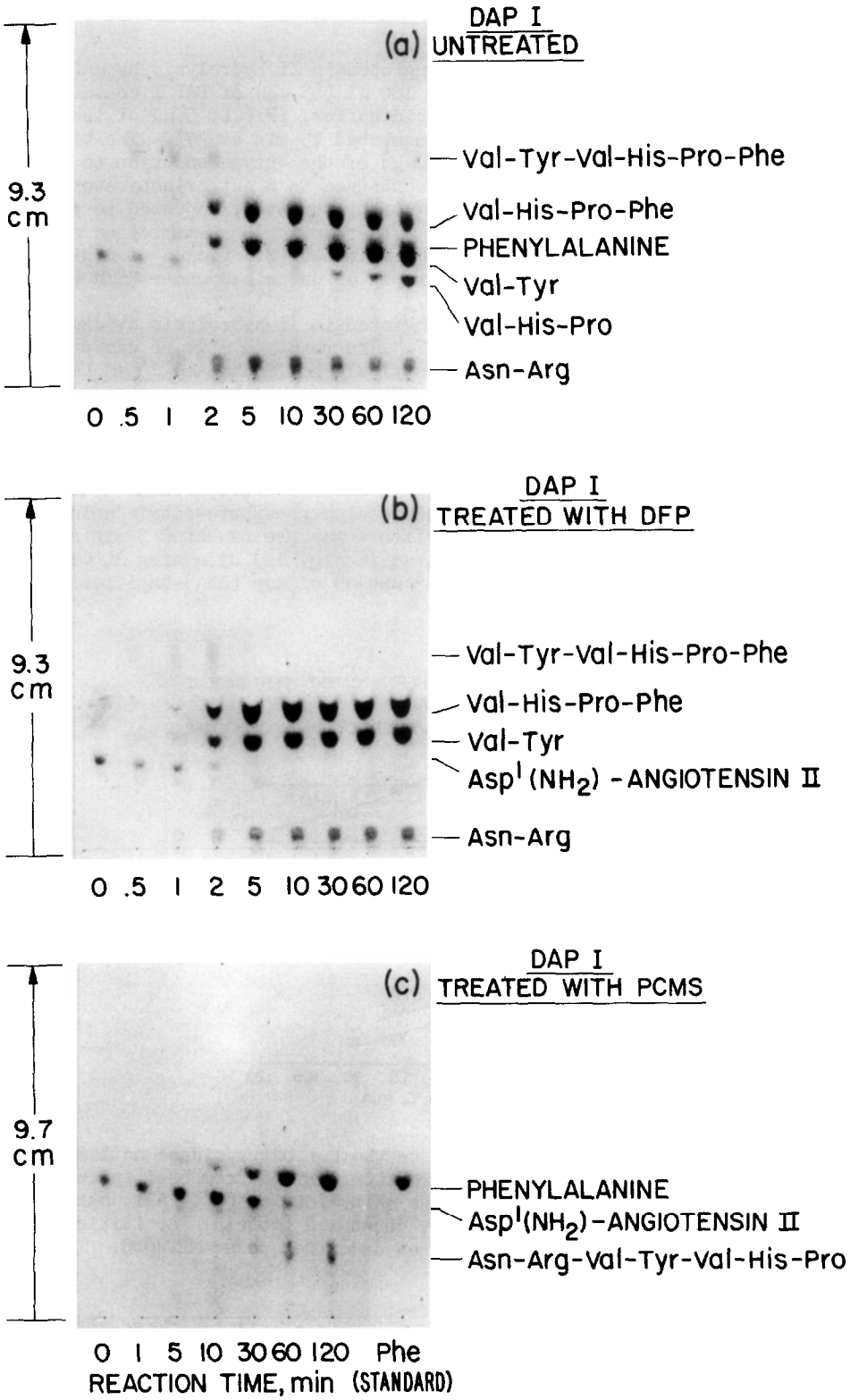

Fig. 1. Effects of inhibitors on the hydrolysis of α-L-Asp^{1}(NH_2)-angiotensin II (Asn-Arg-Val-Tyr-Val-His-Pro-Phe) by highly purified rat liver dipeptidyl aminopeptidase I (cathepsin C).

Fig. 1(a). Products of $Asp^1(NH_2)$-angiotensin II hydrolysis by untreated dipeptidyl aminopeptidase I (DAP I).[2] To 100 µl (65 µg) of DAP I contained in 0.13 M dibasic sodium phosphate-citric acid buffer, pH 6.0, 5 µl of isopropyl alcohol was added, and the mixture was incubated 15 min at 37°. The time course was initiated by adding 20 µl (13 µg) of the enzyme solution to 100 µl (0.3 µmole) of $Asp^1(NH_2)$-angiotensin II contained in a buffer-activator solution comprised of 0.8% pyridine-16 mM HCl-1 mM dithiothreitol, adjusted to pH 5.0 with a negligible volume of acetic acid. The reaction was maintained at 37°. At each time interval, a 1 µl aliquot (equivalent to 2.5 nmoles of angiotensin II) was spotted for thin layer chromatography as described under METHODS.

Fig. 1(b). Products of $Asp^1(NH_2)$-angiotensin II hydrolysis by dipeptidyl aminopeptidase I (DAP I) treated with DFP. The reaction mixture was prepared as described above except that the isopropyl alcohol contained 20 mM DFP. This gave a final DFP concentration of 1 mM during the 15 min preincubation period.

Fig. 1(c). Products of $Asp^1(NH_2)$-angiotensin II hydrolysis by dipeptidyl aminopeptidase I (DAP I) treated with PCMS. DAP I was prepared at a concentration of 1.3 mg per ml in 0.13 M dibasic sodium phosphate-citric acid buffer, pH 6.0, that contained 1 mM PCMS. The mixture was preincubated 5 min at 37°. The time course was initiated (as described in Fig. 1a) by adding 20 µl (26 µg) of the PCMS-treated enzyme to 100 µl (0.3 µmole) of $Asp^1(NH_2)$-angiotensin II solution.

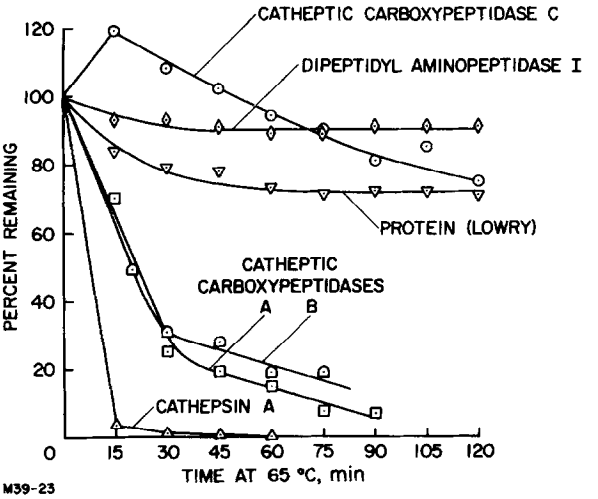

Fig. 2. Effect of heat treatment on the carboxypeptidase activities contained in the DAP I ammonium sulfate fraction derived from beef spleen. A 2% protein solution in 1% NaCl, pH 5.0, was maintained at 65°. Aliquots were removed in time and centrifuged to remove denatured protein. Activities contained in the supernatant were assayed as described under METHODS.

Furthermore, the former activities could not be detected in purified DAP I, and were in fact eliminated in the gel filtration step.

Hydrolysis of Z-Dipeptides - The DAP I peak off Sephadex G-200 was used to study the relative rates of hydrolysis of a variety of Z-dipeptides. Z-Pro-Phe,

a model of the COOH terminus of angiotensin II, was hydrolyzed best around pH 5.5. About 0.1 μmole of Z-Pro-Phe was hydrolyzed per min per mg (Lowry) protein. The hydrolysis of Z-Pro-Phe, like angiotensin II, was strongly inhibited by DFP. Since Z-Pro-Phe-NH_2 and Pro-Phe were not hydrolyzed, the contaminating activity was identified as a carboxypeptidase, here termed "catheptic carboxypeptidase C." Relative to Z-Pro-Phe, rates were also obtained on Z-Pro-Tyr (83%), Z-Pro-Met (75%), Z-Pro-Val (67%), Z-Ser-Met (19%), Z-Pro-Ala (13%), Z-Pro-Ser (5.5%), Z-Glu-Tyr (4%), and Z-Pro-Pro (1.5%). No action was detected on Z-Pro-Gly, Z-Pro-Leu, Z-Phe-Pro, Z-Glu-Phe, and N^{α}-benzoyl-Gly-Arg.

Fate of Catheptic Carboxypeptidase C During the Purification of DAP I - Catheptic CPC accompanied DAP I through the entire purification. As shown in Fig. 2, both activities were exceptionally stable to the standard heat treatment conditions (40 min at 65°). Although the carboxypeptidase was retarded more than DAP I during chromatography on Sephadex G-200, it was not sufficient to effect separation. It was possible, however, during the chromatography of DAP I on DEAE-cellulose, to elute the carboxypeptidase late enough to permit its exclusion from DAP I provided the fractions were discriminately pooled.

Ser-Met Dipeptidase - When catheptic CPC was separated from DAP I on DEAE-cellulose, the Ser-Met dipeptidase activity remained within the DAP I peak. Activities on Z-Pro-Phe and Z-Ser-Met were limited to the carboxypeptidase peak. Ser-Met dipeptidase activity remained with DAP I through the next and final purification step - gradient elution from CM-cellulose.

As shown in Fig. 3, the Ser-Met dipeptidase activity catalyzed the complete hydrolysis of Ser-Met in 2-3 hr. The rate of Ser-Met hydrolysis was only 0.05% of the rate for Gly-Phe-β-naphthylamide. The dipeptidase activity was completely resistant to 1 mM DFP and 0.5 mM PCMS. The activity behaved as a true dipeptidase since the same preparation failed to hydrolyze Ser-Met-NH_2, Z-Ser-Met, Ser-Met-Glu, and a wide range of amino acid β-naphthylamides. The relative rates of hydrolysis on some other dipeptides were Ser-Met > Ser-Ala > Ser-Tyr > Ser-Phe > Ala-Met > Thr-Phe > Met-Ser > Ala-Phe > Ser-Ser. Detectable rates were observed on several others. No activity was detected on Gly-Met, Gly-Phe, Gly-Gly, Gly-Leu, Ser-Pro, and Lys-Lys. Reactions were performed at pH 5.5, which was about the optimum for Ser-Met dipeptidase.

Notably the Ser-Met dipeptidase activity was completely inhibited by 0.5 mM EDTA (Fig. 3). DAP I and catheptic CPC were unaffected by EDTA. The existence of a metal requirement was questionable, however, since the EDTA inhibition was completely relieved following its removal by dialysis (Fig. 3). Furthermore, the addition and subsequent removal of EDTA often resulted in an unmasking of additional (4-fold) dipeptidase activity. Metals such as Hg^{++}, Cu^{++}, Fe^{++} and Fe^{+++} were inhibitors (at 0.5 mM) of Ser-Met dipeptidase. PCMS became inhibitory

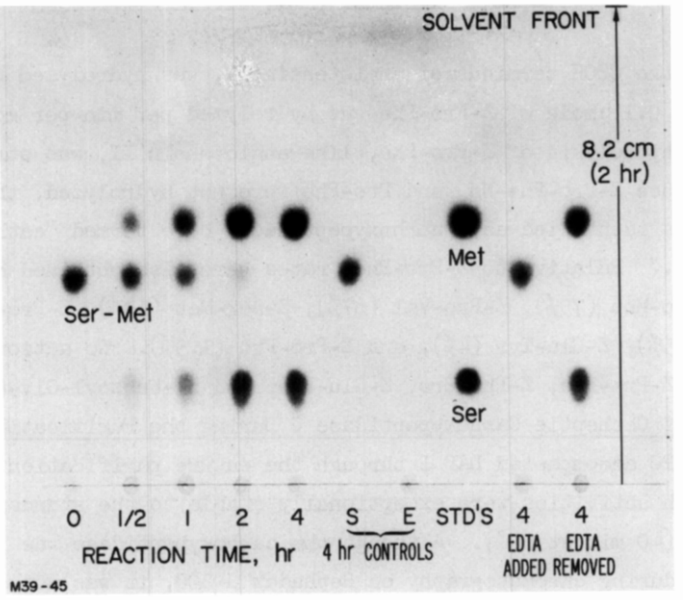

Fig. 3. The hydrolysis of Ser-Met by highly purified (beef spleen) DAP I treated with DFP and PCMS. A 25-ul quantity of 80 mM DFP (in isopropanol) was added to about 50 units (2.5 mg protein) of DAP I contained in 0.5 ml of 0.5% NaCl. The mixture was held on ice for 15 min, combined with an equal volume of 2 mM PCMS in water, and dialyzed overnight against 0.5% NaCl. The inactivation of catheptic carboxypeptidase C and DAP I was confirmed by assay. The time course was initiated by combining 25 µl of the treated enzyme with 25 µl of 6 mM Ser-Met in 1% pyridine-0.45% acetic acid, pH 5.5. At each time interval (0.5, 1, 2 and 4 hr) a 1 µl aliquot was spotted for analysis by thin layer chromatography as described under METHODS. EDTA effects are shown in the last two origins. EDTA was dissolved (at 0.5 mM) into an aliquot of the DFP- and PCMS-treated enzyme, and the mixture was preincubated on ice for 30 min. A 4-hr reaction was conducted on Ser-Met (as above) and compared with another 4-hr reaction conducted with an aliquot of the EDTA-treated enzyme from which the EDTA was first removed by dialysis against 0.5% NaCl.

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at 15 mM, which explained a misleading PCMS effect seen previously (1).

Ser-Met dipeptidase was shown to be an enzyme distinct from DAP I. This was demonstrated by the successful separation by isoelectric focusing of (beef spleen) DAP I (pI = 6.1) and Ser-Met dipeptidase (pI = 5.4) in a pH 5-7 gradient in 1% Ampholine.

Lysosomal Localization - Freshly-excised beef spleen was homogenized in 0.25 M sucrose. A lysosome-rich pellet, prepared by differential centrifugation, was purified by density-equilibrium centrifugation on a continuous gradient of sucrose (30-65 W/V). Catheptic CPC and Ser-Met dipeptidase were located in an isopycnic region (d = 1.188) that was coincident with DAP I, an established lysosomal marker (18,19). Catheptic CPC exhibited its typical DFP sensitivity, and Ser-Met dipeptidase its EDTA sensitivity. Neither activity could be sedimented from aqueous homogenates or from lysosomal fractions treated with Triton X-100.

Preparation of a DAP I Sequencing Reagent - The DAP I fraction recovered from Sephadex G-200 was sufficiently pure to be employed as an effective peptide sequencing tool (3). In this regard, the enzyme is particularly useful when employed in conjunction with methods for the identification of the dipeptide fragments (20). Potential interferences arising from the activities of catheptic CPC and the Ser-Met dipeptidase were eliminated as follows. One part of 80 mM DFP (in isopropanol) was combined with 19 parts of the DAP I pool off Sephadex. The mixture was held on ice for 30 min, concentrated to about 2.5% protein by ultrafiltration (1), and finally dialyzed against 1% NaCl-4 mM 2-mercaptoethanol-0.5 mM EDTA. Aliquots containing 4 mg protein (about 80 units of DAP I) were freeze dried and sealed under vacuum. Between 80 and 90% of the activity survived freeze-drying and was stable for up to two years of storage at 4°. Buffer or saline solutions used to reconstitute DAP I should contain 0.5 mM EDTA to eliminate the activity of Ser-Met dipeptidase. The time course degradation of peptides with DAP I is usually conducted with 3 to 5 units of enzyme per μmole of substrate. It was for this reason that Ser-Met hydrolysis (Fig. 3) was studied at this ratio of DAP I to substrate.

DISCUSSION

Catheptic carboxypeptidase C found in highly purified DAP I was shown to be a distinct and separable enzyme. By way of comparison, the activities of catheptic carboxypeptidases A and B have not yet been shown to be distinct and separable from cathepsin B. A strong resemblance was noted between the properties of catheptic carboxypeptidase C and the lysosomal "angiotensinase" found in kidney by Yang et al. (21). A common enzyme may be responsible for both activities. As part of the present study, and as mentioned previously (1), a potent angiotensinase activity was demonstrated for DAP I.

Ser-Met dipeptidase was also shown to be distinct and separable from DAP I. To our knowledge, all of the dipeptidases thus far reported are metal-dependent enzymes with optimal activity in the neutral-to-alkaline range. None of these activities appear to reside in lysosomes. By contrast, Ser-Met dipeptidase activity has an acidic pH optimum, is lysosomal, possibly lacks a metal requirement, and is resistant to heat and autolytic degradation. It therefore appears that Ser-Met dipeptidase is a new and unusual dipeptidase, and the first lysosomal dipeptidase thus far reported.

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